Unmyelinated Peripheral Nerves can be Stimulated *In Vitro* Using Pulsed Ultrasound

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Abstract

Appreciation for the medical and research potential of ultrasound neuromodulation is growing rapidly, with potential applications in non-invasive treatment of neuro-degenerative disease and functional brain mapping spurring recent progress. However, little progress has been made in our understanding of the ultrasound-tissue interaction. The current study tackles this issue by measuring compound action potentials (CAPs) from an *ex vivo* crab walking leg nerve bundle and analysing the acoustic nature of successful stimuli using a Passive Cavitation Detector (PCD). An unimpeded ultrasound path, new acoustic analysis techniques and simple biological targets are used to detect different modes of cavitation and narrow down the candidate biological effectors with high sensitivity. In the present case, the constituents of unmyelinated axonal tissue alone are found to be sufficient to generate *de novo* action potentials under ultrasound, the stimulation of which is significantly correlated to the presence of inertial cavitation and is never observed in its absence.

Keywords: neurostimulation, neuromodulation, *in Vitro*, peripheral nerves, therapeutic ultrasound, cavitation, Axons

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Introduction

Diseases and dysfunction of the nervous system, both central and peripheral, are common causes of morbidity and mortality around the world. Despite huge investment into pharmaceutical solutions for some of the more prevalent problems, progress has been slow. For a few of these diseases, successful new treatments have been found in neurostimulatory medical devices. Examples include Deep Brain Stimulation (DBS) for Parkinson's disease (Bronstein et al., 2011), Vagus Nerve Stimulation (VNS) for epilepsy and depression (Groves and Brown, 2005) as well as Sacral neuromodulation for incontinence (Thaha et al., 2015). The gold standard for all of these are implantable electrodes, which themselves are associated with much morbidity from the need for highly invasive surgery, regular battery replacements and immunosuppression.

Though implants are improving, techniques that allow non-invasive neurostimulation such as Transcranial Magnetic Stimulation (TMS) (Lee et al., 2012) and Direct Current Stimulation (DCS) (Nitsche et al., 2009) are gaining favour since they avoid the complications mentioned above. However, neither of these techniques can replicate the location specificity, or stimulation of deep structures that implants can achieve.

Ultrasound (US), through the development of High Intensity Focused Ultrasound (HIFU) for ablative surgery and blood brain barrier disruption, has demonstrated its ability to overcome both of these targeting issues, reaching anywhere in the brain and other body areas with millimetre precision. Its application to elicit neuromodulation at lower intensities is still relatively new but is rapidly gaining momentum.

Examples of the neuromodulatory effect of US were first reported as early as 1929 (Harvey, 1929), but surfaced only occasionally until the last decade. These

early, pre-2008 exploratory studies almost all focused on examining effects on peripheral nerves (Fry, 1968; Younan et al., 2013; Sheltawy and Dawson, 1966; Lele, 1963; Gavrilov et al., 1977; Wright et al., 2015; Mihran et al., 1990; Dalecki et al., 1995; Wright et al., 2002; Tsui et al., 2005; Foley et al., 2008) with a few targeting central nervous structures (Tsirulnikov et al., 1988; Wall et al., 1953). This preference shifted dramatically towards central nervous targets after 2008 when Tyler's group demonstrated that hippocampal slices could be stimulated at much lower intensities than those used on peripheral nerves (Tyler et al., 2008). Furthermore, a comparison of threshold neuromodulation intensities in studies on peripheral or central nervous tissue shows the same large difference (Peripheral Nervous System (PNS) mean threshold = 59 W/cm^2 σ = 68 (Fry et al., 1950; Lele, 1963; Gavrilov et al., 1977; Wright and Davies, 1989; Dalecki et al., 1995; Tsui et al., 2005; Foley et al., 2008; Colucci, 2009; Kim et al., 2012; Legon et al., 2012; Dickey et al., 2011; Tych et al., 2013; Lee et al., 2014; Hu et al., 2014), CNS mean threshold = 3 W/cm^2 σ = 3 (Tyler et al., 2008; Tufail et al., 2010; Min et al., 2011b,a,b; Yoo et al., 2011; Moore et al., 2015; Kim et al., 2014a; King et al., 2014; Kim et al., 2015, 2014b; Legon et al., 2014; Lee et al., 2015; Deffieux et al., 2013; Hameroff et al., 2012; Younan et al., 2013; Yang et al., 2012)). Subsequent to 2008, studies on the effects of low intensity US in the living brain have yielded a range of exciting results, such as stimulating motor activity (Tufail et al., 2010), affecting GABA release (Yang et al., 2012), reversibly inhibiting epileptic activity (Min et al., 2011a) and eliciting somatosenory sensations (Lee et al., 2015).

Despite recent progress in the application of the technique, still very little is known about the mechanism at work behind the observations. Understanding in this regard has been hampered by poor characterisation of the ultrasound field, especially in small animal models where small cranial volumes make reflections and

standing waves a significant problem (Young and Henneman, 1961). Combined with the biological complexity of brain tissue and the variety of models used, very little consensus has been achieved on successful US parameters, exemplified by occasional directly conflicting or negative findings (Colucci, 2009; Gavrilov and Tsirulnikov, 2012).

There is at least consensus that ultrasound stimulates nervous tissue through a mechanical effect, not a thermal one. The field is far from united on the nature of this mechanical interaction, but the leading two theories for the key mechanism involve either acoustic radiation force or cavitation.

Cavitation is most often brushed aside as a potential mechanism in the CNS stimulation literature due to the low intensities used to elicit neurostimulation (Tufail et al., 2010; Deffieux et al., 2013; Yoo et al., 2011; Lee et al., 2015), below the FDA recommended Mechanical Index (MI) limits for soft tissue ultrasound (Duck, 2007). The limitations with this claim however are that the MI limit was formulated from observations of bubbles in free water, is concerned only with preventing inertial cavitation of sufficiently large bubbles to cause significant damage, and that MI is only a guide and cannot be used to truly predict the occurrence of cavitation as this will depend on the tissue type, bubble nuclei, dissolved gas content and other factors. Though some studies have reported very high pressure thresholds for *in vivo* cavitation in the brain (Gateau et al., 2011), others have found significant non-inertial cavitation at much lower intensities (240 $\mathrm{mW/cm^2}$) (ter Haar et al., 1982; Ter Harr et al., 1986). Though these two studies had much longer duration exposures of over a minute, the finding does indicate that bubble nuclei can be affected in some way by low intensities over much shorter durations.

In this study, a controlled *in vitro* environment is used, simplifying both the biological and the acoustic environment so that insight can be gained into the mech-

anism by which mechanical forces are transduced into propagating electrical activity in axons. Given this goal, it was decided that the best first course of action was to isolate and understand the direct stimulation phenomena observed previously by the authors in the crab walking leg nerve axon (Wright et al., 2015). To this end, a test setup was designed with several key capabilities:

- Ultrasonic stimulation of a nerve bundle with known exposure parameters.
- Electrical stimulation of the bundle, providing saturated control measurements of the CAP before each US stimulus.
- Measurement of cavitational activity at the US stimulus site.
- Measurement of electrical CAPs at a distal site, resulting from either stimulus modality.

Using this experimental approach combined with modelling of ultrasonic radiation forces at various stimulus parameters, the likely stimulus mechanism was determined by calculating the correlation of radiation force or cavitation activity with successful stimulation. Other features of the successful US stimuli, such as response latency and response reliability, were also investigated to determine the responsible force mechanism.

Materials and Methods

Experimental Setup

The equipment used in the current setup shown in figure 1 is detailed here. US stimulus waveform was produced by two function generators (Agilent 33220A, Agilent, Santa Clara, CA, USA), one gated by the other to produce the pulsed

protocol which was then amplified by a class AB linear power amplifier with 55 dBm gain (E&I 1020L 200 W, E&I , Rochester, NY, USA). The three US stimulus transducers, and the transducer used as a PCD are detailed in Table 1. The signal of the PCD was amplified by a voltage amplifier (SRS inc. Model 445A, Sunnyvale, CA, USA) providing a 5 times gain.

Electrical nerve stimulus was produced using a constant current isolated stimulator (Digitimer DS3, Digitimer, Hertfordshire, UK). Electrical recordings from the nerve were taken using a differential amplifier (WPI DAM50, World Precision Instruments, Sarasota, FL, USA) at 100× DC gain. Electrical and acoustic data was acquired by an oscilloscope (Lecroy HDO6054, 12.5 MHz sampling frequency, Teledyne LeCroy, Chestnut Ridge, NY, USA). Synchronisation of US and electrical stimulation, and signal acquisition was performed using a 4 channel I/O module and DAQ chassis (NI 9402 and NI 9171 cDAQ, National Instruments, Austin, TX, USA).

The nerve bath was separated into three electrically isolated sections. The two ends of the bath performing the electrical stimulation and recording were filled with mineral oil (figure 2) and the middle chamber with a crab ringers solution (525 mmol/L NaCl, 13.3 mmol/L KCl, 12.4 mmol/L $CaCl_2$, 24.8 mmol/L $MgCl_2$ and 5 mmol/L dextrose). All electrodes used for stimulation and recording from the nerve bundle (shown in figure 2) were made from silver chloride coated silver electrodes. To reduce atmospheric electrical noise, the entire setup was contained within a copper mesh Faraday cage.

A deep water bath (20 cm) with an acoustic absorbing layer (figure 1) was used to prevent ultrasound reflection interfering with the US field at the focal point. Reflections within the water bath were measured to affect the peak focal pressure by less than 5% at any of the amplitudes used in this study. The water bath was cooled with ice and monitored to ensure that it stayed between 1-4 °C. The cold slows down

the nerve's rate of conduction which serves to separate its response from stimulation artefacts and keeps the nerve viable for longer. The focused PCD was fixed in place within the water bath at an angle and distance such that its focal zone overlapped the focus of the stimulus transducer on the nerve bundle and such that it avoided receiving the direct field of the stimulus transducer (figure 1).

Nerve Preparation Procedure

All nerves were taken from live crabs (*Cancer pagurus*) sourced on the day of use from London markets. As invertebrates, crabs are not subject to regulatory requirements on animal testing in the UK. Nerves were extracted from the crab leg by stripping away each joint section, removing the shell and muscle from around the nerve bundle, leaving as much as possible of the nerve intact. During extraction the nerve was regularly sprayed with chilled (4-10 °C) crab ringer's solution. The nerve was then ligated at both the proximal and distal ends with red cotton thread. Cutting above the distal ligation, the nerve was detached from the claw, transferred into the nerve bath and wetted with chilled saline. The nerve was handled by the string attachments and passed through the two blocking gates (figure 2), then pulled straight between them. This ensured that the nerve was located directly under the ultrasonic focus (within $\pm$ 0.1 mm). Oil was then added to the two side channels and saline to the centre. Surface tension in the small aperture of the blocking gates (most of which was occluded by the nerve diameter) prevented the oil and saline from mixing between the chambers.

Once loaded, the chamber was transferred to a holder on the surface of the water bath and an US coupling cone fitted on top, ensuring that no bubbles were trapped in the US propagation path using a small endoscopic camera viewing from below. The nerve bath and all implements were cleaned and sterilised with ethanol before

use.

Nerve Bundle Characterisation

Five extracted nerve bundles were fixed in 3% glutaraldehyde (0.05 mol/L sodium cacodylate buffer pH 7.2-7.4) directly after extraction. The bundle was then sectioned and fixed in araldite resin using a methylene blue/azure II/ basic fuchsin stain for light microscopy examination. Axon fibre density was estimated using a digitally applied, randomly positioned 50 μm square, counting only the axonal cells with more than 50% of their volume within the square. This was repeated three times for each of the 5 nerve bundles. Neuron density was calculated to be 136/100 μm (σ =27), combined with the mean cross-sectional area of a nerve bundle the total number of nerve axons in an extracted bundle was found to be 1017 (σ =202).

Investigation into the cause of the increased likelihood of initial response success found in a previous study by the authors (Wright et al., 2015), led to the observation of microbubbles on the surface of the nerve bundle by light microscopy. These bubbles are introduced by the extraction process as the bundle is submerged into the saline bath. In the 0-5 minute period post nerve submersion, a mean of 11 bubbles (σ=8.8, n=10) with a mean diameter of 78 μm (σ=54) were seen over 2 cm of nerve. The microbubbles were not observed past the first two US stimuli of an experiment as larger bubbles were observed to rise to the surface after US exposure and smaller ones dissolved rapidly into the surrounding saline. As only the first couple of stimuli are affected by these bubbles, it was decided not to degas the nerve ringers solution, as this would cause the axons to die faster.

Ultrasound Setup

To produce a highly predictable experimental US field a good understanding of the field and focus produced by each ultrasound transducer was required. The

free field spatial pressure distribution of the three HIFU transducers used in the experiments (Table 1) were measured using fibre-optic hydrophones (plane tipped, 10 μm diameter, calibrated frequency range of 500 kHz to 50 MHz) in a degassed water tank.

The spatial, temporal peak pressures for each transducer were located in 3 dimensions and measured at relatively small peak negative pressure amplitudes (0 to -2 MPa) by three fibre-optic hydrophones, taking the mean positive and negative pressures for each transducer at three different input powers. Mean measurements from multiple hydrophones were used to minimise inaccuracy from sensitivity variation between different probes. Larger pressure amplitudes were not directly measured due to the risk of damage to the hydrophones and therefore inaccurate measurements, as per the manufacturer's recommendations (limited to <3 MPa at 1 MHz). Instead, the measured pressure values were used to parametrise a Khokhlov-Zabolotskaya-Kuznetzov (KZK) based model of acoustic fields for each transducer, changing the output efficiency parameter to match the measured outputs. This model takes into account non-linear effects by modelling the propagation of the first 50 harmonics around the fundamental frequency. The model was then used to predict peak negative pressures of exposures below -2 MPa used in the current study (Table 2). The KZK model has been validated using low f-number transducers similar to the ones used in this study (Canney et al., 2008). Furthermore, radial peak positive pressure at the focal point of each transducer was obtained by solving the calibrated KZK, and was confirmed to be within a 10% tolerance of hydrophone measured profiles.

Rigid ultrasound coupling cones were machined from perspex for each transducer that both sealed in degassed water for near field transmission and mechanically locked onto the nerve bath. These fixed the focal point along the central axis, 5 mm beyond the end of the cone. The apertures of the cones were set at 20 mm, much larger

than the diameter of the 1st side lobe (measured using a fibre-optic hydrophone in degassed water to be 15.2 mm in diameter to the 2nd nul point for the 0.67 MHz transducer 5 mm before the focal point). The truncated end of these cones was sealed with thin mylar film (12 μm), providing an acoustically transparent window into the nerve bath. The wide top of the cones fitted each transducer tightly, preventing leakage of water and lateral targeting errors. The presence of the cone, affixed to each transducer was found to have no measurable effect on the dimensions or peak positive pressure of the focal points, measured in a degassed water tank.

Thin mylar film was also used as an acoustic window in the nerve bath, separating the water in the cone from the nerve bath, and the saline in the nerve bath from the water bath underneath. The width of the acoustic window in the nerve bath was 10 mm.

Ultrasound targeting error was analysed by producing visible heating spots in thermo-chromatic gels with each transducer (figure 3). The centre point of the colour change and its lateral deviation from the centre line of the chamber were measured three times for each transducer, dismantling and re-constructing the apparatus each time. Deviation was found to be a maximum of 160 ($\sigma = 67$), 84 ($\sigma = 11$) and 89 μm ($\sigma = 40$ μm) for 0.67, 1.1 and 2 MHz respectively. As the errors are much smaller than the width of the nerve (1-2 mm), a portion of the nerve bundle will always be exposed to the focal maximum.

Ultrasound and Electrical Stimulation Protocols

Electrical stimulation of the nerve bundle was performed to provided a measurement of maximum CAP amplitude and conduction speed, monitoring the health of the bundle and allowing the proportion of the bundle stimulated by US to be determined. Electrical and US stimulation were paired in these experiments so that

every US stimulus was preceded by an electrical stimulus, 3 seconds apart (a CAP in this chilled and unmyelinated model lasts 100 ms). The timing between each electrical and US stimulus pair was alternately varied between 30 and 90 seconds, causing the whole pattern to repeat every 120 seconds with an average of 1 stimulus pair per minute (figure 4a). Each nerve bundle was exposed to 22 of these stimulus pairs resulting in a total experimental time of 22 minutes. The paired pulse protocol was designed to investigate if recovery times had an effect on either the cavitation environment (i.e. on the presence of cavitation nuclei) or biological environment.

Electrical stimulation was applied via the stimulation electrode (figure 2a) using a 0.2 ms constant current pulse. Stimulation amplitude was adjusted before each experiment to achieve saturation. Full saturation may not have been achieved every time due to varying levels of saline short between the stimulation electrodes and the earthed central bath. Larger crabs with generally thicker nerve bundles were preferentially selected to reduce this effect, as their nerve bundles better occluded the holes in the blocking gates.

Ultrasound parameters were initially chosen based on precedence in the literature for successful neurostimulation protocols (King et al., 2013; Kim et al., 2014a; Tufail et al., 2010). Variation and optimisation of these stimulus parameters in preliminary experiments (data not shown) led to a novel protocol described below.

The primary stimulus protocol used in this study was, 80 pulses of 0.67 MHz driving frequency at 10 kHz Pulse Repetition Frequency (PRF), over an 8 ms Total Stimulus Duration (TSD) (50% duty cycle). Short duration stimuli (8 ms) were chosen to ensure temporal separation of the electrical noise artefact from the received electrical nerve signal. Intensity was varied between the values shown in table 2. Orders of intensities being tested on a single nerve were randomised to prevent systematic error from nerve inhibition or other effects. The 1.1 and 2 MHz exposure

parameters shown in table 2 were calculated to match the radiation forces produced by the 0.67 MHz exposures.

To test the effect of longer exposure durations on the nerve response dynamics at 0.67 MHz, a second set of stimulus experiments, with the same parameters as above, were performed using 100 ms instead of 8 ms stimulus durations (1000 pulses).

To test the effect of different pulsing protocols on stimulation success without exploring the entire parameter space, the parameters found in a recent successful *in vivo* US neurostimulation study by Lee et al. were tested (250 kHz fundamental, 500 Hz PRF, 50% duty cycle for 300 ms, with 3 s between each stimulus (Lee et al., 2015)) at the higher frequency of 0.67 MHz in our current setup. These parameters were used initially at 0.7 W/cm^2, shown to be effective in Lee's study, and then incrementally increased in the same steps seen in table 2 until a response threshold was found. As with all exposure protocols, each stimulus intensity was repeated 22 times on a new nerve bundle.

KZK modelling determined that there was significant non-linear propagation of ultrasound at the power levels used in the current study, increasing at higher amplitudes at all frequencies (Table 2). This results in higher positive pressures compared to the peak negative values. To facilitate easy comparison with other papers in the literature, Spatial Peak Pulse Average Intensity (I_{SPPA}) will be used throughout the rest of the paper but it should be noted that these are linear approximations and the positive and negative pressure peaks will be the most accurate metrics, especially at higher amplitudes. These peak pressures are displayed along side peak and pulse average intensity values at all frequencies and driving powers (Table 2).

Detection and Analysis of Electrical Signals

To detect CAPs, the electrical signal was split into 10 ms windows with no overlap and an FFT performed on each. As the extracellular population recording of a CAP is a summation of many cells' ionic fluxes in the environment around the nerve bundle, the low frequency component (0-5 MHz) was used for detection. An amplitude threshold for CAP detection was set at 5 times the standard deviation (σ) of the low frequency background activity or 5σ of the total background noise in the time domain. The integrated area under the curve of a CAP, 5 ms each side of the peak voltage amplitude was used to measure the response amplitudes for both electrical and US stimulated CAPs.

The, electrically stimulated CAP response was used as a reference point to normalise the absolute amplitudes recorded from US stimulation.

CAP response latency was measured from the onset of the ultrasound or electrical stimulus to the peak of the resultant CAP. This was the median latency of all the fibres in the bundle which includes US travel, nerve response and CAP transmission time. The first of these was constant and calculated to be 47 μs and 40 μs for the 0.67 and 1.1/2 MHz transducers respectively. The CAP transmission time along the nerve was estimated on a nerve by nerve basis using the preceding CAP transmission time from the interleaved electrical stimuli and the known relative distances between the electrodes. Subtracting these from the total lag time, an estimate for the US response latency was calculated for each. This method assumes that the stimulation occurs in the centre of the US focal point every time, uniform conduction velocity along the length of the nerve and that the relative position of the CAP peak amplitude does not change with time or different stimulation modalities.

The US transducers may induce noise in the recording electrodes (Francis et al., 2003). Though this source of noise was greatly reduced by using an earthed saline

bath, a temporal and spectral filtering algorithm was designed to prevent such noise being mistaken for CAP responses.

Analysis of PCD Signals

PCD recordings were analysed to determine the presence of inertial cavitation, which is characterised by a high energy, short duration, broadband signal. Analysis of time domain signal spikiness (kurtosis) and energy (variance) in multiple frequency bands was therefore used to detect inertial cavitation events.

In cases where multiple cavitation events are occurring simultaneously, as was usually the case in this study, smaller amplitude events can be difficult to detect and quantify using standard methods (Chen et al., 2003; Tu et al., 2006). A multi-resolution signal processing method is used here which demonstrates a promising performance for this application (Haqshenas and Saffari, 2015). The technique uses the wavelet transformation to decompose the signal into several components across the following frequency ranges: $\frac{f_N}{2^{n+1}} - \frac{f_N}{2^n}, n = 0, \ldots, M-2$, where f_N is the Nyquist frequency (6.25 MHz) and M is the levels of decomposition (5 levels).

After performing the discrete wavelet transformation, short Fourier transform (STFT) and statistical analysis (i.e. variance and kurtosis) of each component of the signal are carried out to identify and characterise different cavitation regimes (Haqshenas and Saffari, 2015). Inertial cavitation is indicated by a high value of time domain kurtosis. The kurtosis threshold was set using the standard deviation of kurtosis in the lowest amplitude exposures as a baseline noise measurement, as no inertial cavitation was observed in standard spectrographic analysis. A kurtosis threshold for cavitation detection was therefore set at 6.

In the case of the 100 and 300 ms exposures, the key 10 ms section of the US stimulus likely to have caused any resultant CAP, was determined by subtracting

the expected CAP transmission lag time from the point when the CAP peak was received. A 10 ms section of PCD signal data was analysed around the resulting time point, illustrated by the vertical red lines in (figure 4c). In cases where no US stimulated CAP was detected, a random 10 ms time section of PCD data was analysed for comparison.

Calculation of the Acoustic Radiation Force

Radiation forces produced by the 0.67 MHz stimulation protocol shown in table 2 were calculated by summing the force caused by acoustic absorption within the nerve, with the force caused by acoustic reflection from the surface of the nerve. The former is calculated as follows (Leighton, 1994):

$$I_{\mathrm{SPPA}} = \frac{P_{ac}^2}{\sqrt{2}Z}, \qquad (1) \qquad\qquad F_{abs} = \frac{2\alpha I_{\mathrm{SPPA}}}{c}, \qquad (2)$$

where P_{ac} is the peak pressure, Z is the characteristic acoustic impedance of brain tissue (1.6 MRayls), F_{abs} is the radiation force due to the absorption of acoustic energy, α is the absorption coefficient of neural tissue in neppers per meter, calculated with the equation:

$$\alpha = \alpha_0 f^y, \qquad (3)$$

where f is frequency, y is the frequency dependence exponent (an exponent of 1.3 and α_0 of 8.6 for brain tissue was used (Duck, 1990)) and c is the speed of sound in soft tissue (1562 m/s) (Roy, 1991). These equations assume plane wave and linear propagation.

Radiation forces due to the reflection at the saline-tissue interface were then calculated (Leighton, 1994):

$$R = \frac{Z_2 - Z_1}{Z_2 + Z_1}, \qquad (4) \qquad\qquad F_{ref} = \frac{2 I_{SPPA} R}{c}, \qquad (5)$$

where R is the pressure reflection coefficient, Z_1 and Z_2 are the specific acoustic impedances for saline and tissue respectively and F_{ref} is the radiation force acting on the boundary due to reflection assuming a linear plane wave, perpendicular angle of incidence and a reflecting surface area much larger than the wavelength. For the purposes of this summation, the difference between the planes upon which the force was acting was assumed to be negligible due to the small dimensions of the nerve.

These equations were used to calculate the radiation forces produced by the 0.67 MHz exposures and find the focal intensities required at 1.1 and 2 MHz to produce identical forces. This resulted in a range of 17-475 W/cm^2 at 1.1 MHz and 12-343 W/cm^2 at 2 MHz (Table 2).

Damage Detection

Damage to the nerve bundle was detected by two means. The primary method was to measure proportional reductions in electrical stimulation amplitude from one stimulation to the next. A significant damage event detected by these means was defined as more than a 20% reduction caused by a single US stimulus. This threshold was defined by 1.5 times the maximum point to point decline detected in nerves not exposed to US. Three such control nerves were recorded over 22 minutes using the same experimental protocol but without power to the US transducer.

Damage that may have been caused by US stimulation over a longer period was tested for by determining the correlation coefficient of the decline in CAP amplitude with acoustic kurtosis and signal energy at all frequency bands in each nerve experiment. Correlation was also tested for between the same variables, irrespective of US frequency or individual nerve experiments, across each stimulation protocol.

The second method used to detect significant damage events was through identification of after-discharge (repetitive nerve activation) after a successful US stimulation event. After-discharge is known as a sign of poration in the nerve membrane as the charged ions equilibrate causing the membrane to regularly depolarise (Lee et al., 1995). After-discharge was identified when the standard deviation of the raw electrical signal (100-150 ms after the CAP peak) was more than 1.5 times greater than the background σ measured before CAP initiation.

Results

142 nerves responsive to electrical stimulation were exposed to a range of ultrasound parameters which were shown to be capable of eliciting large, synchronous CAP events from the unmyelinated crab leg nerve bundle (figure 4b). Responsive nerve bundles could be stimulated multiple times in the same location, with stimulus reliability varying between 5 and 80%, strongly depending on fundamental frequency and pulse average intensity of stimulation. Nerve responses occurred unpredictably at different US exposures throughout the 22 minutes of an experiment, however, there was an increased response probability for the first stimulus (15% of all experiments responded on the first attempt compared to a mean of 7% success for any other of the 22 stimuli).

The lowest intensity at which stimulation was observed was at 100 W/cm^2 I_{SPPA} for the 8 ms, 0.67 MHz stimulus. Inertial cavitation signals were detected in all

successful stimuli and found to be significantly correlated with nerve responses in the 100 ms, 0.67 MHz stimulus experiments in all frequency bands (P <0.05). The results from each stimulus protocol variant are presented in this section.

Direct stimulation of the nerve via the electric field was ruled out by a sham experiment where the US cone was raised, creating a reflecting air gap between the cone and the saline bath and the primary US stimulation protocol repeated at high intensity. No direct stimulation was observed in this manner across 3 electrically responsive nerves and 66 individual stimuli (562 W/cm^2, 8 ms TSD, 0.67 MHz, 10 kHz PRF, 50% duty cycle, 30/90 s repetition period).

8 ms 0.67 MHz Stimuli

61 electrically responsive nerves across 26 crabs were tested using a range of 11 different US stimulation intensities (Table 2). Nerve response reliability and amplitude for each intensity stimulus are shown in figure 5. signal energy and kurtosis of the PCD data are shown in figure 6. The overall response reliability was less than 25% at all intensities (figure 5a).

The lowest intensity where neurostimulation was observed was 100 W/cm^2. Correlation coefficients between the amplitude of the CAP response and both PCD signal energy and kurtosis, find significant (P<0.05, n=22) correlation in two nerve experiments (out of 61) at 485 and 562 W/cm^2 across all frequency bands. Mean response latency was 3.16 ms (n=106), measured from stimulation onset and excluding the time taken for the CAP to reach the recording electrodes.

Inertial cavitation was found to be ubiquitous at pulse average intensities past 100 W/cm^2, with broad band (1.56-6.25 MHz) inertial events (kurtosis>6) occurring in more than 70% of US stimuli (figure 7). This matches with the threshold for successful US stimulation also seen at 100 W/cm^2. The majority of these cavitation

events are not associated with any resultant nerve activity.

100 ms, 0.67 MHz Stimuli

19 electrically responsive nerves across 6 crabs were tested using the same range of US stimulation intensities (Table 2) as used in the 8 ms protocol. Nerve response reliability and amplitude for each intensity stimulus are shown in figure 8a and b respectively with the PCD signal kurtosis shown in c. The lowest intensity where neurostimulation was observed was 169 W/cm^2.

Significant positive correlation (P <0.05) between nerve response amplitude and cavitation measures (kurtosis and signal energy of key PCD time sections) was found in 5 individual nerve experiments (56% of US responsive nerve experiments) across all frequency bands.

All cavitation and nerve response data in the 100 ms exposures was aggregated to determine the correlation, irrespective of US driving intensity and separate nerve experiments. Significant positive correlation was found between kurtosis of acoustic signals and nerve response amplitude across all frequency bands (0.39-0.78 MHz: $r = 0.25$ $P < 0.005$, 0.78-1.56 MHz: $r = 0.23$ $P < 0.005$, 1.56-3.13 MHz: $r = 0.18$ $P < 0.05$, 3.13-6.25 MHz: $r = 0.2$ $P < 0.005$, n=304). This strongly implicates the involvement of inertial cavitation. In individual STFT and wavelet analysis of the PCD data from every successful US stimulation, inertial cavitation signals were found in the expected time section without exception.

Similar to the 8 ms exposures, ubiquitous cavitation activity detectable in all frequency bands was found over 169 W/cm^2 shown in figure 8c. However, Analysis of the kurtosis of separate time sections showed that the majority of the inertial activity was restricted to the first 10 ms time bin (figure 9). Some events did occur after the initial burst of cavitation such as shown in figure 4c, at a much lower event

frequency, demonstrated by the much lower mean kurtosis values seen in figure 8c compared to figure 6b.

300 ms 0.67 MHz Stimuli

Reproductions of the intensities and pulse parameters found in CNS stimulation literature (Lee et al., 2015) at 0.67 MHz were unable to generate CAP responses in the crab nerve bundle. Incrementally increasing the intensity of stimulation resulted in a threshold for CAP generation at 169 W/cm^2.

1.1 and 2 MHz Stimuli

58 electrically responsive nerves across 11 crabs were tested using a range of 5 different US stimulation intensities that equalled the radiation forces produced in the 0.67 MHz exposures (Table 2). At these intensity values, no nerve responses were observed.

Occasional high kurtosis events were seen with 1.1 and 2 MHz exposures, though the acoustic signal energy in frequency bands other than driving was near zero (<0.1% of total signal energy at all points) with very low standard deviation between experiments. Therefore the cavitation activity present was considered negligible.

Damage

US was found to damage the exposed nerve bundles in some cases. The lowest intensity example of after-discharge in the 0.67 MHz 8 ms protocol, at 230 W/cm^2, is shown in figure 10c. Two other after-discharge events in separate nerve experiments were observed above this intensity threshold (at 485 and 562 W/cm^2), all three were concurrent with reduced electrically stimulated CAP amplitude.

Each of the damage events causing after-discharge were examined spectrographically. Large broadband noise signatures marking inertial cavitation events (figure 10d) were seen preceding all these instances of significant damage. The cause was therefore deemed likely to be inertial cavitation induced membrane rupture.

Significant positive correlation ($P<0.05$, $n=22$) between decline of the electrically stimulated CAP and acoustic kurtosis & signal energy was seen in two of the three after-discharge occurrences mentioned above and in three more nerve experiments at 419 W/cm^2. Positive correlation is also found in 3/19 nerve experiments in the 100 ms exposures. In total, damage was observed in 4% of all nerve experiments at any intensity or frequency. No significant damage as a result of US exposure occurred at either 1.1 or 2 MHz.

Sham experiments were performed on three nerves from one crab where no US was used. Degradation of the electrically stimulated CAPs across 22 minutes (Mean normalised decline per minute = 0.009, $\sigma=0.013$) was not significantly different from mean decline of US exposed nerve bundles, where signs of major damage events as above were not seen at any intensity. The rate of decline in CAP amplitude over the 22 minutes also did not significantly change between exposure intensities (figure 10b). No significant correlation between damage and either signal energy or kurtosis was found when measured across all data for each stimulation protocol, irrespective of US frequency or individual nerve experiments.

Discussion

Our results demonstrate that unmyelinated axonal tissue alone is sufficient to generate *de novo* action potentials in response to ultrasound stimulation. Examining the nature of this response allows several insights into the underlying mechanisms, which, in the present case, the authors demonstrate to be cavitational.

The lowest threshold at which responses were seen in any of the experiments conducted here, was an order of magnitude greater than pulse average intensities used in some studies achieving successful stimulation in rat brain tissue (Kim et al., 2012, 2014b,a; Tufail et al., 2010; Yoo et al., 2011). These studies use lower frequency ultrasound (250-350 kHz) which has indeed been shown to be a critical factor by the current study and others (King et al., 2013; Kim et al., 2014a; Gavrilov et al., 1977; Muratore et al., 2009; Lee et al., 2014) which may account even for this large discrepancy in the pulse average intensity threshold. From investigations repeating the pulsing parameters of an applied study in the human brain (Lee et al., 2015), pulse protocol does not appear to play a role. The mechanism observed here and in many *in vivo* brain studies may be the same but given the very different cavitation environments, until further research can be performed the mechanisms should be treated as distinct. Subsequent discussion will therefore focus on the characterisation of the currently observed stimulation phenomenon.

The intensity thresholds found in this study are much closer to those reported by Gavrilov's group and others targeting peripheral nerve structures (Gavrilov et al., 1996; Wright and Davies, 1989; Mihran et al., 1990; Legon et al., 2012; Tych et al., 2013), re-enforcing the apparent divide in threshold amplitude between neuromodulation of the CNS and the PNS. The extent of the separation in required intensities between these two paradigms demonstrate the importance of identifying in different tissue types, the specific US effects and their thresholds. This could then be used to develop a fuller understanding of the US-tissue interaction and targeted ultrasound therapies, including but not limited to neurostimulation.

Ultrasound Force Mechanism

Response Dynamics

The first thing that was noted about the nerve responses was the stochastic success rate, where large events that involved many axons in the bundle occur infrequently. This points to a correspondingly probabilistic cause that occurs on a scale affecting a large proportion of the fibres in the bundle or not at all, consistent with cavitating bubbles occurring outside of nerve fibres. If the mechanism of stimulation was on a small scale such as the bilayer sonophore model (Krasovitski et al., 2011), the many isolated events that act at the individual cell level would be expected to produce a reliable response when aggregated to the level of the entire bundle.

The same argument against cellular scale probabilistic effects can be applied to discount a radiation force mechanism. In a system where nothing is being changed between US exposures, radiation force as a result of tissue absorption and reflection should remain constant as well as any effects on the nerve it elicits, but this is not what was observed. Second to this, the radiation forces produced in the 0.67 MHz exposures were calculated and reproduced at 1.1 and 2 MHz (Table 2) and found to be ineffective at generating responses from the nerve. Indeed these modelled forces are lower than compressional experiments in the literature shown to generate mechanical stimulation of axons (Rivera et al., 2000), though conductance changes from weak compression may contribute to the overall effect (Julian and Goldman, 1962; Olesen et al., 1988).

The response latency dynamics of the current observed phenomena also does not match with studies that find only the onset or offset of US stimuli to be effective (Menz et al., 2013; King et al., 2013; Gavrilov et al., 1977; Dalecki et al., 1995; Krasovitski et al., 2011; Plaksin et al., 2014) which would be consistent with radiation

force or bilayer sonophore mechanisms. In the current findings, stimuli occurrences are distributed throughout the 8 and 100 ms exposures, with each part of the pulse train having a similar chance of stimulating the nerve.

PCD Data

In both the 8 ms and 100 ms datasets a plateauing of kurtosis and signal energy are seen after 230 W/cm^2 (figure 6). This is likely to be caused both by the non-linear scaling of peak negative pressures with intensity (Table 2), and saturation in the occurrence (but not amplitude) of cavitation. Concurrently with this observation, response reliability also saturates around this intensity (figure 5 and figure 8). Therefore increasing US intensity past a point will not increase the likelihood of stimulation and may increase the violence of events and likelihood of damage.

Across all US stimuli, inertial cavitation was most often observed with no resultant nerve response. The probable reason for this is that the focal area (6 $\times$ 3.5 mm FWHM) and potential volume in which cavitation is likely to occur, was much larger than the volume occupied by the nerve bundle (1-2 mm diameter). Cavitation therefore may not be occurring in close proximity to the nerve.

This affected the analysis of the 8 ms much more than the 100 ms exposures as the non-proximal cavitation activity is found throughout the 8ms exposure and only in the first 10 ms of longer stimuli. This lower average background activity over the longer exposure period led to significant correlations between US stimulated CAP amplitude and PCD signal kurtosis in individual nerve experiments and across the whole dataset.

Correlation coefficients can mask infrequent stimulation events that may occur without any sign of cavitation. A key finding of this paper therefore, is that, through detailed individual PCD signal analysis, broad frequency band inertial cavitation

events were detected in the expected time section preceding 100% of successful US neurostimulation events in both the 8 and 100 ms datasets.

Damage

Damage was found to occur as a result of US exposure in several cases, strongly correlated to inertial cavitation at all intensities. The lowest instance of damage occurring close to the threshold (230 W/cm^2) for stimulation raises concerns as to the safety of US stimulation at these intensities. However, given the present scope for optimisation and refinement of the stimulation parameters, it is hoped that the risk of damage can be eliminated in future. It is also as yet unclear how a full, *in vivo* situation will affect both the success of the stimulation effect and the occurrence of damage but the latter should be examined in more depth *in vitro* before applying the current technique to animal models.

Biological Mechanism

Given the nature of the causative US forces discussed above and the presence of axonal tissue alone, the authors suggest that the mechanism of membrane depolarisation has been narrowed down to two options. The first option involves the opening of ion channels by membrane stretch induced by cavitational forces such as microstreaming drag, direct jetting, or radiation forces on bubbles. The second is general ionic flux and resultant depolarisation through a sonoporation effect caused by the same cavitation mechanisms (Wan et al., 2015).

Responses that were followed by after-discharge (figure 10c) from identified damage events were likely due to large scale membrane perforation or tearing that resulted from inertial cavitation forces. It is hoped that through planned future work using high speed imaging and computational bubble modelling, the critical biological interaction can be determined and a force threshold identified.

Conclusions

Reported here are successful parameters for ultrasonic neurostimulation in the peripheral crab leg nerve bundle, demonstrating that the constituents of unmyelinated axonal tissue are sufficient to generate *de novo* action potentials in response to US stimulus, in the majority of cases without lasting damage. The threshold for this stimulation was much higher than similar procedures performed on CNS models but in good agreement with other PNS focused studies. Low intensity stimulation parameters shown to be successful *in vivo* in the literature were unsuccessful at generating any response from the nerve bundles. Given the difference in threshold intensities, the current observed stimulation phenomenon is assumed to have a distinct US force mechanism.

In characterising the observed stimulation phenomena, inertial cavitation activity was found to be highly correlated to successful US stimulation, with its acoustic signature present in every example. With further work into protocol refinement and control of cavitation nuclei, this US stimulation mechanism will have incredible potential for both clinical and research applications. Future work by the group will aim to determine the exact cellular level forces required to generate stimulation in this and other models.

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Figure Captions

Figure 1: Schematic diagram of the experimental setup used to generate alternate electrical and US stimuli recordings.

Figure 2: Schematic diagram of the nerve bath with electrodes and important features labelled.

Figure 3: Visualisation of the size and position of the focal points produced by each ultrasound transducer within the nerve bath (a) 0.67 MHz (b) 1.1 MHz (c) 2 MHz. Temperature induced colour changes were produced in an polyacrylamide gel. 10s continuous wave exposures with different focal intensities were used with each transducer to achieve a good visualisation of the focal area.

Figure 4: (a) Timeline of the interleaved US and electrical stimulation protocol. Electrical stimulation is marked by vertical black lines, US stimulation by vertical blue lines. Each US stimulus is comprised of a pulse train of 0.5 ms pulses at 50% DC. The entire stimulus protocol is repeated every 2 minutes 11 times for every nerve experiment. (b&c) Example of a CAP (electrode voltage data - red, left axis. Shown by a small deviation from the mean) which was stimulated by an ultrasound pulse (b) 0.67 MHz, 8 ms, 562 W/cm^2 c) 0.67 MHz, 100 ms, 562 W/cm^2). PCD data showing the US stimulus is included above the electrode voltage data (acoustic amplitude - blue, right axis). High amplitude acoustic signal containing cavitation signatures are detected at the start of both stimuli. (c) Vertical red lines show the period of the acoustic pulse train within which the stimulus event is expected to have occurred given estimated CAP conduction times. Increased acoustic signal amplitude containing cavitation signatures is seen within this period.

Figure 5: Response success statistics for the 8 ms, 0.67 MHz stimulation protocol. Nerves that did not respond to electrical stimuli were excluded. Numbers of successful stimuli at each intensity are displayed above each bar. (a) Total response reliability for all nerves tested at each intensity level. Two nerve experiments with over 50% reliability were excluded as outliers. (b) Mean amplitude of US induced CAPs as a proportion of the electrically induced CAP amplitude.

Figure 6: Mean and standard deviation error bars of (a) signal energy and (b) time domain kurtosis in four frequency bands decomposed from PCD recordings of the 8 ms, 0.67 MHz US stimulation protocols. The frequency band containing the US driving frequency is highlighted in red.

Figure 7: Percentage of US stimuli (8 ms,0.67 MHz protocol) showing above threshold kurtosis (>6) in four frequency bands at each stimulus intensity. In each frequency band, 11 columns are present representing the different stimulus intensities. The colourmap on the right is in units of W/cm^2.

Figure 8: ((a) and (b)) Response success statistics for the 100 ms, 0.67 MHz stimulation protocol. Nerves that did not respond to electrical stimuli were excluded. Numbers of successful stimuli at each intensity are displayed above each bar (a) Mean response reliability for all nerves tested at each intensity level. (b) Mean Amplitude of US induced CAPs as a proportion of saturated electrical stimulus recording taken before each US stimuli. (c) Mean acoustic signal kurtosis of four frequency bands across all intensities. Error bars show standard deviation.

Figure 9: Mean values of PCD signal kurtosis of the lowest frequency band over

100 ms, split into 10 ms divisions and a range of stimulation intensities (I_{SPPA}).

Figure 10: (a) Example of electrically stimulated CAP amplitude (line) and US stimuli (+) over 22 stimuli on a single nerve. This example was exposed using the 0.67 MHz, 8 ms stimulation protocol at 562 W/cm^2. (b) Mean decline over time of the electrically stimulated CAP amplitude of the 0.67 MHz, 8ms stimulated nerves, normalised to the amplitude of the first stimulus with standard deviation error bars. (c) Example of after-discharge due to nerve damage using the 0.67 MHz, 8 ms stimulation protocol at 230 W/cm^2. (d) spectrographic analysis of the PCD signal of the first damage causing ultrasound event in (a).

Table 1: My caption

Model	Manufacturer	CF (MHz)	Focal Length (cm)	Aperture (cm)	LFA (cm)	WFA (cm)
PA409	Precision Acoustics	0.67	7.2	6.0	4	0.5
H-101-MR	Sonic Concepts	1.1	6.3	6.4	1	0.14
H-106	Sonic Concepts	2	6.3	6.4	0.6	0.08
XL50PCD	Ultran	5.8	7.7	1.3	-	-

Tables

Table 1: HIFU transducer reference table. CF = Centre Frequency, LFA = Length of Focal Area, WFA = Width of Focal Area. Focal area dimensions are given according to the FWHM. The sensitivity bandwidth of the XL50 PCD was 4.8 MHz at -6 dB with a bandwidth centre frequency of 6.8 MHz.

Table 2: Intensities, negative and positive peak pressures and radiation forces at three frequencies. Intensities and pressures were chosen to create equal radiation forces across the frequencies used.

	0.67 MHz			1.1 MHz			2 MHz		
Radiation force (mN/cm^2)	Pressure (MPa)		I_{SPPA} (W/cm^2)	Pressure (MPa)		I_{SPPA} (W/cm^2)	Pressure (MPa)		I_{SPPA} (W/cm^2)
1	-0.8	0.8	20	-0.7	0.7	17	-0.7	0.5	12
5	-1.4	1.6	76						
6	-1.6	1.8	100	-1.5	1.7	84	-1.4	1.3	61
9	-1.9	2.2	140						
11	-2.1	2.4	169	-1.9	2.2	143	-1.7	1.8	103
15	-2.4	2.9	230						
18	-2.6	3.2	274	-2.4	2.8	232	-2.1	2.4	167
23	-2.8	3.6	352						
27	-3.1	4.0	419	-2.9	3.6	353	-2.5	3.0	255
31	-3.3	4.3	485						
36	-3.5	4.7	562	-3.3	4.2	475	-2.8	3.6	343